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Vitamin A and immune regulation: Role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance

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Abstract

The vitamin A (VA) metabolite all-*trans* retinoic acid (RA) plays a key role in mucosal immune responses. RA is produced by gut-associated dendritic cells (DC), on which it also acts in a positive feedback loop to induce enzymes involved in its own synthesis. RA is required for generating gut-tropic lymphocytes and IgA-antibody-secreting cells (IgA-ASC). Moreover, RA modulates $Foxp3^+$ T_{REG} and Th17 differentiation. Thus, although recent evidence indicates that RA could be used as an effective "mucosal adjuvant" in vaccines, it also appears to be required for establishing intestinal immune tolerance. Here we discuss the roles proposed for RA in shaping intestinal immune responses and tolerance at the gut mucosal interface. We also focus on recent data exploring the mechanisms by which gut-associated DC acquire RA-producing capacity.

Keywords

vitamin A; retinoic acid; homing; dendritic cells; tolerance

1. Introduction

VA deficiency is an important public health problem, particularly in developing countries where it is associated with increased susceptibility to gastrointestinal and lung infectious diseases, poor response to vaccination, increased HIV pathogenesis, and overall increased mortality, especially in children (Sommer et al., 1983; Villamor and Fawzi, 2005). Accordingly, VA supplementation can dramatically reduce child mortality in these settings (Sommer et al., 1986; Villamor and Fawzi, 2005; West et al., 1991).

The VA metabolite RA is responsible for most of the biological effects of VA (Theodosiou et al., 2010). RA plays essential and pleiotropic roles in bone formation, reproduction, and organogenesis during embryonic development (Mark et al., 2006). Moreover, RA fulfills important functions in the formation of epithelial linings of the skin and mucosal tissues,

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which act as barriers to the external environment (McCullough et al., 1999; Wang et al., 1997). In addition, as we will discuss below, RA affects the innate and adaptive immune system in a number of ways (Iwata, 2009; Mora et al., 2008; Stephensen, 2001).

A seminal paper published by Iwata and collaborators in 2004 showed that RA is critical for inducing lymphocyte trafficking to the intestinal mucosa and that DC from Peyer's patches (PP) and mesenteric lymph nodes (MLN) (gut-associated DC), but not from extra-intestinal tissues, can metabolize VA into RA (Iwata et al., 2004). Subsequent work showed that RA was also required to induce gut tropic B cells and to promote the differentiation of IgA-ASC in mice and humans (Mora et al., 2006). Furthermore, xRA was also shown to modulate $Foxp3^+$ regulatory T cell (T_{REG}) and Th17 cell differentiation (Benson et al., 2007; Coombes et al., 2007; Kang et al., 2007; Mucida et al., 2007; Schambach et al., 2007; Sun et al., 2007; Wang et al., 2010). Thus, vitamin A is important for both intestinal immune responses to pathogens and tolerance to food antigens and commensals. Disrupted RA signals, causing altered homing or impaired functional differentiation of lymphocytes, might be implicated in diseases such as inflammatory bowel diseases, type I diabetes, food allergy, and some infectious diarrhea.

Here we focus on the mechanisms regulating RA production by gut-associated DC and on the role of RA and gut-tropic lymphocytes in intestinal immune homeostasis and tolerance.

2. Vitamin A metabolism

VA is usually acquired from the diet either as all-*trans* retinol, retinyl esters, or β-carotene (Napoli, 2011; Theodosiou et al., 2010). All-*trans* retinol is esterified to retinyl esters and stored in the liver or it can associate to retinol binding protein (RBP), which transports retinol to target tissues (Napoli, 2011; Theodosiou et al., 2010). All-*trans* retinol is then oxidized intracellularly to all-*trans* retinal by ubiquitously expressed retinol dehydrogenases (RDH), which belong to the short chain dehydrogenase reductase (SDR) gene family. At least three RDH seem to be physiologically involved in this rate-limiting step: RDH1, RDH10 and DHRS9 (Napoli, 2011). Then, cytosolic retinal dehydrogenase enzymes (RALDH) catalyze the irreversible oxidation of all-*trans* retinal to RA (Napoli, 2011; Theodosiou et al., 2010).

At least four RALDH enzyme isoforms (RALDH1, RALDH2, RALDH3, and RALDH4) have been identified in mice, and highly homologous enzymes are present in humans and other chordates, indicative of the physiological importance of RA metabolism for many organisms. Genetic deletion experiments allowed to analyze the respective physiological contribution of the various RALDH to RA production (Penzes et al., 1997). Whereas $RALDH1^{-/-}$ mice are viable (Fan et al., 2003), $RALDH2^{-/-}$ and $RALDH3^{-/-}$ mice show early lethality, suggesting that these enzymes play essential roles in RA production during development (Dupe et al., 2003; Niederreither et al., 2003). RALDH4 has been cloned in mice, but its physiological contribution to retinoid metabolism remains to be determined (Lin et al., 2003).

RALDH expression is restricted to limited cell types. In adult mammals, three RALDH isoforms have been described in gut-associated cells, including small- and large-intestinal epithelial cells (IECs), mesenteric lymph nodes (MLN) stromal cells, and gut-associated DC (DC from Peyer's patches, small-intestinal LP and MLN). IEC express RALDH1 (Bhat, 1998; Frota-Ruchon et al., 2000; Iwata et al., 2004; Lampen et al., 2000), whereas stromal cells in MLN express RALDH2 and probably RALDH1 and RALDH3 (Hammerschmidt et al., 2008; Molenaar et al., 2011). Among gut-associated DC, PP-DC express RALDH1 and to a lower extent RALDH2, whereas MLN-DC only express RALDH2 (Coombes et al., 2007; Iwata et al., 2004; Jaensson et al., 2008; Yokota et al., 2009). However, as we will

discuss below, the relative *in vivo* relevance of RA production by different types of gut-associated cells, as well as the functional implications of expressing different RALDH isoforms, remain to be fully determined.

RA exerts its effects mostly through binding to heterodimers of nuclear RA receptors (RARα, β, γ) and retinoid X receptors (RXRα, β, γ) (Samarut and Rochette-Egly, 2011), although some specific effects can be mediated via PPARβ/γ (Mora et al., 2008; Schug et al., 2007). RAR-RXR heterodimers are ligand-dependent transcription factors that bind to *cis*-acting DNA sequences, called RA response elements (RARE), located in the promoter region of RA target genes. Although RAR receptors are ubiquitously expressed, RARβ expression is markedly enhanced by RA (Samarut and Rochette-Egly, 2011).

Termination of RA signaling is achieved through its catabolism into oxidized metabolites, such as 4-hydroxy RA and 4-oxo RA, by enzymes of the CYP26 family. Among these enzymes, CYP26A1 is directly upregulated by RA (Samarut and Rochette-Egly, 2011). While RA metabolites have been mostly considered to be biologically inert, there is evidence indicating that some of these metabolites retain the ability to signal through RAR (Sorg et al., 2008; Theodosiou et al., 2010). However, potential roles of RA metabolites in immune responses have not been described.

3. Role of RA in the regulation of T and B cell immune responses

3.1 RA reciprocally regulates the induction of gut- and skin-tropic lymphocytes

Lymphocyte migration to different lymphoid and extra-lymphoid tissues requires the expression of specific receptors on lymphocytes *(homing receptors)* and their corresponding ligands *(addressins)* on endothelial cells from tissue postcapillary venules (Mora, 2008). Naïve T and B cells recirculate among different lymphoid compartments and once they are activated by their cognate antigen they acquire the capacity to migrate to extra-lymphoid tissues (Mora, 2008). The skin and the gastrointestinal (GI) mucosa are the largest surfaces in the body exposed to the external environment and are also the extra-lymphoid tissues with the best-characterized migration requirements. Homing to the skin requires the expression of P-/E-selectin ligands, chemokine receptor CCR4, and integrin αLβ2 (LFA-1) on T cells as well as their respective ligands P-/E-selectin, CCL17/TARC, and ICAM-1 expressed in skin postcapillary venules (Mora, 2008). In contrast, migration to the small intestine lamina propria (LP) relies on integrin α4β7 (LPAM-1) and chemokine receptor CCR9 on T and B cells and their respective ligands, i.e., mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25/TECK in small bowel postcapillary venules. Of note, homing to the large bowel LP requires the integrins α4β7 and α4β7, but not CCR9 (Mora, 2008). In addition, it should be kept in mind that alternative pathways of lymphocyte recruitment to the intestine may occur in steady state as well as during inflammatory conditions (Villablanca et al., 2011a).

Regarding how lymphocytes acquire tissue-specific migratory capacity upon activation, an important observation was that gut-associated DC induce gut-homing receptors α4β7 and CCR9 on T cells upon activation, thus endowing activated T cells with gut-tropism (Johansson-Lindbom et al., 2003; Mora et al., 2003; Stagg et al., 2002). In addition to demonstrating for the first time a tissue-specific factor responsible for directing T cell migration, these studies highlighted a completely new function for DC, aside from their well-known role in T cell activation.

From a mechanistic standpoint, a groundbreaking advance in the field was the discovery that RA is sufficient and also necessary to induce gut-homing receptors on T cells (Iwata et al., 2004). RA is also required, and sufficient, to induce gut-tropic IgA-ASC in mice and

humans (Hammerschmidt et al., 2011; Mora et al., 2006; Uematsu et al., 2008) as well as gut-homing human T cells (Eksteen et al., 2009) (Figure 1). Accordingly, some gut-associated DC, but not extra-intestinal DC, express high levels of RALDH enzymes and can synthesize RA, which is required for their gut-homing imprinting capacity. Indeed, induction of gut homing receptors on activated T cells occurred only when retinol, the substrate to synthesize RA, was provided to gut-associated DC (Coombes et al., 2007; Iwata et al., 2004; Yokota et al., 2009).

The *in vivo* evidence demonstrating the importance of RA for inducing gut-tropic lymphocytes was gained from studies performed in vitamin A (VA)-depleted (VAD) mice. In order to generate VAD mice, pregnant females received a purified diet that lacked VA starting from days 7–10 of gestation. The pups were weaned at 4 weeks and were maintained on a VAD diet until 12 weeks of age before analysis was performed (Iwata et al., 2004; Mora et al., 2006). Mice deficient in lecithin:retinol acyltransferase (LRAT), which cannot store retinol in the liver, provide a convenient alternative to achieve VA depletion in a shorter time-frame and hence avoid potential unwanted effects of chronic VA depletion (O'Byrne et al., 2005). $LRAT^{-/-}$ mice develop normally when maintained on a VA sufficient diet, but they become VAD after only 2–4 weeks on a VAD diet (O'Byrne et al., 2005). VA depletion can be readily attested by the sharp reduction in the levels of the free retinol and retinyl esters in adipose tissue (O'Byrne et al., 2005).

Importantly, VA-deficient (VAD) mice lack T cells and IgA-ASC in the small intestine but not elsewhere (Iwata et al., 2004; Mora et al., 2006) and the induction of gut-tropic effector T cells is abrogated in VAD mice (Hall et al., 2011; Jaensson-Gyllenback et al., 2011; Villablanca et al., 2011b). The mechanism by which RA induces α4β7 and CCR9 in T cells is mediated, at least in part, by a direct effect of RA/RARα on *Itga4* (encoding α4 integrin chain) and *Ccr9* gene promoters (Hill et al., 2008; Kang et al., 2011; Ohoka et al., 2011). Interestingly, in addition to inducing gut-homing receptors, gut-associated DC and RA block the upregulation of skin-homing receptors on T lymphocytes (Iwata et al., 2004; Mora, 2008). Thus, RA reciprocally regulates the differentiation of gut- and skin-homing lymphocytes.

In addition to gut-associated DC, murine MLN stromal cells ($CD45^{Neg}MHCII^{Neg}CD11c^{Neg}gp38^{+}$), can also produce RA and are sufficient to imprint gut-tropic T and/or B cells *ex vivo* (Hammerschmidt et al., 2008; Molenaar et al., 2011). Nonetheless, whether RA production by DC and/or stromal cells is required *in vivo* for imprinting gut-homing lymphocytes has not been determined. Given that depletion of these cell types will likely affect lymphoid architecture and/or lymphocyte activation, cell-specific knockouts for RALDH enzymes will be required to clarify this issue.

3.2 Role of RA in IgA production

Secretory IgA (SIgA) contributes to the intestinal barrier function and recent evidence supports the notion that such antibodies are involved in immunological homeostasis (Mantis et al., Muc Immunol 2011). Production of SIgA depends on IgA-antibody secreting plasma cells (ASC) and their immediate precursors (plasmablasts), which accumulate in the mucosa by selective homing mechanisms among which RA play an important role.

VAD rats or mice have decreased levels of total SIgA in intestinal lavages and decreased mucosal antigen-specific IgA responses, which have been correlated with impaired protection against oral infections and bacterial toxins (Mora et al., 2008; Sirisinha et al., 1980; Wiedermann et al., 1993). Of note, although VAD animals exhibited decreased numbers of IgA-ASC in the small bowel (Bjersing et al., 2002; Mora et al., 2006), their

serum IgA levels were normal (Mora et al., 2006), suggesting that RA is not required for IgA-ASC differentiation in other mucosal compartments.

In addition to its role in physiological IgA-ASC differentiation, RA is sufficient to induce IgA production in *ex vivo* LPS-activated splenocytes (Tokuyama and Tokuyama, 1993; Tokuyama and Tokuyama, 1996). In this line, gut-associated DC induce IgA-ASCs by a mechanism depending, at least in part, on RA (Chang et al., 2008; Massacand et al., 2008; Mora et al., 2006; Uematsu et al., 2008), however other factors, including IL-5, IL-6 and TGF-β, are required for IgA class-switching and plasma cell differentiation (Sato et al., 2003; Tokuyama and Tokuyama, 1999; Watanabe et al., 2010) (Figure 1).

Interestingly, a recent report proposed that follicular dendritic cells (FDC), a subset of stromal cells found in lymphoid follicles, are necessary for IgA production in PP and that RA and TLR signals are required to confer FDC with IgA-inducing capacity (Fagarasan et al., 2010). In addition, plasmacytoid DC (pDC; $CD11c^{int}B220^{+}PDCA1^{+}$) were proposed to be the main DC subset in charge of inducing thymus-independent IgA in PP and MLN via a mechanism involving APRIL, but not RA (Tezuka et al., 2011). However, although pDC were sufficient to induce IgA *in vitro*, whether these cells are necessary *in vivo* for IgA production remains to be determined. It is also unclear whether FDC and DC might interact or complement each other for IgA production.

3.3 RA modulates T_{REG} and Th17 differentiation

While the intestinal mucosa needs to elicit protective immune responses against pathogenic microorganisms, inflammatory immune responses to food and harmless commensal microbiota should be prevented. In fact, T cell activation by oral antigens in the absence of inflammation favors naïve T cell differentiation to $Foxp3^{+}$ regulatory T cells (T_{REG}), which are crucial to prevent deleterious immune responses in the gut mucosa.

Initial studies showed that RA potentiates TGF-β-dependent T_{REG} induction, while it reciprocally inhibits pro-inflammatory Th17 differentiation *ex vivo* (Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007). In addition, RA confers T_{REG} with gut-homing capacity by inducing α4β7 and CCR9 (Benson et al., 2007; Kang et al., 2007; Moore et al., 2009; Siewert et al., 2007). Although it is possible that RA directly acts on T cells for T_{REG} differentiation (Nolting et al., 2009; Xiao et al., 2008a), RA might also contribute indirectly by blocking suppressive signals provided by memory T cells and/or antigen-presenting cells (e.g., pro-inflammatory or Th2 cytokines that inhibit T*REG* induction) (Hill et al., 2008; Takaki et al., 2008).

While the *in vitro* effects of RA on T_{REG}/Th17 are consistently reported in different studies, the *in vivo* effects and physiological roles of RA on T_{REG}/Th17 differentiation are much less clear and seem to vary significantly depending on the experimental system. For instance, one study showed that RA supplementation did not affect T_{REG} induction, while it blocked pathogenic Th17 cells in experimental allergic encephalomyelitis (EAE) (Xiao et al., 2008b). Conversely, data in a diabetes model showed that RA increased T_{REG} without affecting Th17 cell frequencies (Van et al., 2009).

Interestingly, although RA enhances T_{REG} differentiation *in vitro*, VAD mice did not show a decrease in T_{REG} frequencies (Cha et al., 2010; Kang et al., 2009) and T_{REG} from VAD mice efficiently suppressed inflammation in a mouse model of ileitis (Kang et al., 2009). However, the latter study did not assess whether RA was required in the recipient mice to suppress inflammation upon donor T_{REG} transfer. Similarly, although *in vitro* data show that RA acts via RARα to induce T_{REG} (Hill et al., 2008; Schambach et al., 2007), $RAR\alpha^{-/-}$ mice showed normal T_{REG} levels in the lamina propria (Hall et al., 2011; Hill et al., 2008).

A caveat in the interpretation of the results described above is that it was not systematically discriminated between naturally occurring/thymus-derived T_{REG} (nT_{REG}, $CD4^+Foxp3^+Helios^+$) and adaptive/inducible antigen-specific T_{REG} (iT_{REG}, $CD4^+Foxp3^+Helios^{Neg}$). In fact, whereas nT_{REG} were not affected, *de novo* iT_{REG} induction upon oral immunization was significantly abrogated in VAD mice (Hall et al., 2011).

On the other hand, despite the effect of RA blocking *in vitro* Th17 differentiation, RA induced gut-homing receptors on Th17 cells and VAD mice exhibited a marked decrease in Th17 numbers in the small bowel (Cha et al., 2010; Wang et al., 2010). Moreover, RA was required for Th1 and Th17 differentiation during intestinal *Toxoplama gondii* infection (Hall et al., 2011) and during an experimental model of celiac disease (DePaolo et al., 2011). Thus, RA appears to be critical *in vivo* for inducing and/or maintaining Th17 cells in the intestinal mucosa under steady-state and at least during some inflammatory settings. Alternatively, RA might be required to induce gut-homing Th17 cells, but not extra-intestinal pro-inflammatory Th17 responses. In fact, whereas high RA concentrations block Th17 induction, low RA levels seem to be required to induce Th17 cells *in vitro* (Uematsu et al., 2008), Analogously, low RA concentrations might allow the *in vivo* induction of gut-homing Th17 cells (Cha et al., 2010) (Figure 1).

Given the discrepancies mentioned above between *in vitro* and *in vivo* results regarding the effects of RA on T_{REG}/Th17 responses, it will be important that future studies routinely analyze antigen-specific iT_{REG}, nT_{REG}, and Th17 responses, ideally in intestinal and extra-intestinal compartments. In addition, it should be kept in mind that RA deficiency might also affect epithelial integrity and the gut microbiota. In this regard, *Segmented filamentous bacteria* (SFB) were significantly reduced in the ileum of VAD mice (Cha et al., 2010). Since SFB induce intestinal Th17 differentiation (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009), decreased SFB might also partially explain the decrease in intestinal Th17 cells in VAD mice.

Among RA sources in the intestinal mucosa are gut-associated DC (Iwata et al., 2004), LP macrophages (Denning et al., 2007), IEC (Bhat, 1998; Frota-Ruchon et al., 2000; Lampen et al., 2000), and stromal cells (Hammerschmidt et al., 2008; Molenaar et al., 2009). Although all these cell types might potentially affect T_{REG}/Th17 balance in the gut, DC are likely to play a determinant role in these responses. In fact, in addition to synthesizing RA, $CD103^+$ DC express αvβ8 integrin (critical to activate latent TGF-β), and specific deletion of αv or β8 integrin chains in DC significantly impaired T_{REG} (Paidassi et al., 2011; Worthington et al., 2011) and Th17 (Acharya et al., 2010; Melton et al., 2010) responses.

3.4 Role of RA in Th1 and Th2 polarization

VA deficiency correlates with decreased Th2 responses (Wiedermann et al., 1993), while VA supplementation blocks Th1 and promotes Th2 differentiation *in vitro* and *in vivo* (Iwata et al., 2003; Nikawa et al., 1999). While this Th2-promoting effect might favor protection in the gut mucosa by increasing humoral immune responses (Nikawa et al., 1999), it might also cause a predisposition to Th2-associated pathologies, such as asthma (Schuster et al., 2008). Mechanistically, RA promotes Th2 differentiation by inducing *Gata3*, *Maf*, *Stat6*, and *Il4* genes, while reciprocally inhibiting *Tbet* expression (Dawson et al., 2006; Iwata et al., 2003; Lovett-Racke and Racke, 2002) (Figure 1). RA might also indirectly exert a Th2-promoting effect through modulation of DC (Hoag et al., 2002). It will be interesting to determine whether RA interacts with other DC-modulating factors for inducing Th2 responses, such as thymic stromal lymphopoietin (TSLP) (Ito et al., 2005; Rimoldi et al., 2005), IL-33 (Rank et al., 2009), or Notch ligands (Amsen et al., 2004; Maekawa et al., 2003).

4. Origin and differentiation of RA-producing DC

During the past years, several studies highlighted the presence of phenotypically distinct gut-specific APC subsets, which express macrophage and DC markers. Emerging evidence suggest that RA plays a significant role in regulating the functions of APC in intestinal immune compartments. Moreover, RA is produced by many APC subsets and might differentially modulate the induction of Th1, Th2, Th17, and T_{REG} responses.

4.1 DC subsets in intestinal LP

Two major populations of murine phagocytic mononuclear subsets have been identified in the steady-state intestinal mucosa: $CD11c^{+}CD103^{+}CX3CR1^{Neg}$ (also $CD11b^{+/Neg}CD8\alpha^{+/Neg}F4/80^{Neg}$) DC and $CD11c^{+/Neg}$ $CD103^{Neg}CX3CR1^{+}$ (also $CD11b^{+}CD8\alpha^{Neg}F4/80^{+}$) macrophage-like cells, hereafter referred to as $CD103^{+}$ and $CX3CR1^{+}$ subsets, respectively (Denning et al., 2011; Yokota et al., 2009). While macrophage/DC precursors (MDP) give raise to both $CD103^{+}$ DC and $CX3CR1^{+}$ macrophages, common dendritic cell precursors (CDP) and pre-DC give raise only to $CD103^{+}$ DC. On the other hand, $LyC6^{high}$ monocytes only produce $CX3CR1^{+}$ macrophages (Bogunovic et al., 2009; Varol et al., 2007; Varol et al., 2009). In addition, $CD103^{+}$ DC development depends on Flt3L (Bogunovic et al., 2009; Varol et al., 2007; Varol et al., 2009) whereas $CX3CR1^{+}$ macrophages depend on M-CSF (Bogunovic et al., 2009). Whether all murine DC/macrophage subsets have phenotypic and functional counterparts in humans remains to be elucidated. Nonetheless, most $CD103^{+}$ gut-associated DC express high levels of *Aldh1a2* mRNA (encoding RALDH2) in mice and humans, hence they are able to synthesize RA, induce gut-tropic T cells, and promote TGF-β-dependent T_{REG} differentiation (Coombes et al., 2007; Jaensson et al., 2008; Sun et al., 2007). Moreover, only $CD103^{+}$ DC transport antigens from intestinal LP to the MLN, where they activate cognate antigen-specific naïve T cells (Schulz et al., 2009). In contrast, LP $CX3CR1^{+}$ macrophages express *Aldh1a1* (encoding RALDH1) (Denning et al., 2011) and exhibit significantly lower RA producing capacity than $CD103^{+}$ LP DC in the small intestine, which correlates with a decreased gut-homing-inducing ability (Denning et al., 2011; Schulz et al., 2009). Interestingly, a subset of human lamina propria macrophages expressing CD14 and CD209 and which have been proposed to play a pro-inflammatory role in Crohn's disease (CD), also express *ALDH1A2* (Kamada et al., JI 2009).

$CX3CR1^{+}$ macrophages are characterized by their close association with IEC, with some of them sending dendrites through the epithelial layer to sample antigens from the intestinal lumen (Niess et al., 2005). However, despite the fact that $CX3CR1^{+}$ macrophages can capture gut-borne antigens, these cells exhibit slow turnover and remain stationary in the LP, without migrating to the MLN (Schulz et al., 2009). How $CD103^{+}$ DC acquire intestinal antigens to activate T cells in MLN remains to be clarified, although it is possible that these cells get antigens indirectly via IEC and/or from $CX3CR1^{+}$ macrophages.

4.2 How are gut-associated DC educated to produce RA?

Although it is well demonstrated that some gut-associated DC express RALDH enzymes and therefore are specialized in producing RA, it was important to understand how DC are "educated" in this tissue-specific functional property. Are bone marrow (BM) DC precursors pre-committed to become RA-producers or are they educated locally in the intestinal mucosal environment?

Since some DC are in close contact with IEC (Niess et al., 2005), it was proposed that these cells educate DC with gut-associated functional properties. In this regard, extra-intestinal DC co-cultured with IEC, or IEC lines, induced T_{REG} (Iliev et al., 2009a; Iliev et al.,

2009b), Th2 cells (Rimoldi et al., 2005) and gut-tropic T cells (Edele et al., 2008). Such *ex vivo* DC education required RA, TGF-β, and/or TSLP production by IEC (Edele et al., 2008; Iliev et al., 2009a; Rimoldi et al., 2005), both in mice and humans. However, whether, and how, IEC condition gut-associated DC *in vivo* remains to be demonstrated. In fact, $CX3CR1^{+}$ DC do not acquire RA-producing capacity despite being in close contact to IEC (Schulz et al., 2009), suggesting that contact with IEC is not sufficient to educate gut-associated DC or that different DC subsets have different conditioning requirements.

Of interest, GM-CSF was shown to be sufficient to induce *Aldh1a2* in extra-intestinal murine DC and mice lacking the common β subunit of GM-CSF/IL-3/IL-5 receptor exhibited impaired gut-associated DC education (Yokota et al., 2009). However, gut-associated DC from $GM\text{-}CSF^{-/-}$ mice were not impaired in their RA-synthesizing or gut-homing inducing potential (Wang et al., 2011), suggesting that this cytokine might not be essential for gut-associated DC education.

Among other possible candidates for DC education, PPARγ−agonists induced RA-synthesizing enzymes in human DC (Szatmari et al., 2006), but the results in murine DC are less clear (Housley et al., 2009; Villablanca et al., 2011b). In addition, LXR-ligands induced *Aldh1a1* and *Aldh1a2* in liver cells but not in DC (Huq et al., 2006; Villablanca et al., 2011b), suggesting that the expression of RALDH enzymes is differentially regulated among different tissues. On the other hand, PGE2, which is produced by skin stromal cells, inhibits *Aldh1a2* expression in skin-derived DC (Stock et al., 2011). However, blocking prostaglandin synthesis by using cyclooxygenase (COX)-inhibitors markedly decreased *Aldh1a2* expression in MLN-DC in the context of ileitis (Samson et al., 2011), suggesting that the effect of prostaglandins on RA production by DC might vary depending on the tissue analyzed and/or inflammatory status.

Interestingly, a recent report proposed that the Wnt/β-catenin pathway is involved in gut-associated DC education to induce tolerogenic responses in the gut mucosa. Several Wnt signaling proteins are expressed in LP-DC and specific deletion of β-catenin in DC resulted in markedly decreased expression of *Aldh1a2* and RA-synthesizing capacity by gut-associated DC, which correlated with decreased induction of Treg and exacerbated inflammation in DSS colitis (Manicassamy et al., 2010).

4.3 Role of RA and TLR signals in gut-associated DC differentiation

Gut-associated DC are exposed to RA in the small intestine LP, and RA levels correlate with the ability of DC to induce gut tropic T cells and Treg (Villablanca et al., 2011b). In line with these observations, recent work by several groups supports the hypothesis that RA induces its own synthesis in DC in a positive feedback loop. RA is sufficient *in vitro* and *in vivo* to induce *Aldh1a2* in extra-intestinal DC (e.g., PLN-DC), conferring them with RA-producing and gut-homing imprinting capacity, both in mice and humans cells (Hammerschmidt et al., 2011; Villablanca et al., 2011b) (Figure 2). Yokota *et al.* initially showed that MLN-DC from VAD mice exhibited markedly impaired RA-producing capacity (Yokota et al., 2009). These results were reproduced and extended by showing that gut-associated DC from VAD mice induced lower levels of gut-tropic lymphocytes, T_{REG} and IgA-ASC compared with their counterparts from mice on a VA-sufficient diet (Feng et al., 2010; Jaensson-Gyllenback et al., 2011; Molenaar et al., 2011; Villablanca et al., 2011b; Yokota et al., 2009). Of note, MLN stromal cells from VAD mice also expressed low *Aldh1a2* mRNA levels, suggesting that RA is also required for MLN stromal cell education (Molenaar et al., 2011). Although clear evidence demonstrates the physiological importance of RA for *in vivo* DC and stromal cell education, the molecular mechanism by which RA induces its own synthesis in the target cells is not fully understood. However, the lack of retinoic acid response elements (RARE) in the *Aldh1a2* gene promoter, the requirement for

de novo protein synthesis, and the delayed kinetic of protein transcription compared to known RA-target genes (Villablanca et al., 2011b) suggest that RA might induce *Aldh1a2* transcription indirectly.

If RA is necessary *in vivo* to induce *Aldh1a2* expression in gut-associated DC, hence conferring them with RA-synthesizing capacity, it raises the "chicken-and-egg" question of which cells are the initial source of RA which then educates DC in the gut. IEC express *Aldh1a1* (Iliev et al., 2009a; Lampen et al., 2000) and can produce RA (Frota-Ruchon et al., 2000; Iwata et al., 2004). Moreover, *Aldh1a1* expression in IEC does not decrease in VAD mice (rather it increases) (Bhat, 1998; Frota-Ruchon et al., 2000), suggesting that IEC might be hard-wired to synthesize RA, or that their RA-producing capacity depends on other environmental factors (e.g., TLR signals, Wnt/β-catenin pathway, or microbiota). Thus, IEC might provide, at least in part, a primary supply of RA in the gut mucosa, which might act like a "spark-plug" in gut-associated DC to induce in these cells a positive feedback loop driving *Aldh1a2* expression. Experiments using *in vivo* cell-specific *Aldh1* and/or *Aldh2* deletion might be needed to answer these questions.

Interestingly, a subset of BM cells also express *Aldh1a2* (Feng et al., 2010), suggesting that RA might also be produced in the BM microenvironment, perhaps pre-conditioning DC precursors with RA synthesizing capacity prior to their arrival in the gut mucosa. Of note, RA induced CCR9 on *in vitro*-generated BM-DC, while CD103 was not upregulated in BM-DC by RA treatment (Feng et al., 2010). This is in agreement with a lack of RA requirement *in vivo* for CD103 expression in gut-associated DC (Villablanca et al., 2011b). Moreover, MLN-DC from CD103$^{-/-}$ mice were not impaired in inducing gut-tropic T cells (Jaensson et al., 2008), suggesting that although CD103 is a useful marker for identifying RA-producing DC, it is not required for DC intestinal education and/or activity.

In the steady state, *Aldh1a2* is induced in MLN-DC as well as in MLN stromal cells after birth and its expression increases throughout life (Molenaar et al., 2011), suggesting that postnatal exposure to the external environment might be required for conferring gut-associated DC with RA-synthesizing capacity. In this regard, the intestinal microflora plays a major role in maintaining normal immune homeostasis in the gut (Hooper and Macpherson, 2010), and microbial-associated molecular patterns, in particular TLR-agonists, modulate DC function in a number of ways. The TLR5-ligand flagellin increased *Aldh1a2* expression in LP-DC and induces IgA-ASC in a RA-dependent manner (Uematsu et al., 2008). Moreover, the TLR2-ligand zymosan induced *de novo Aldh1a2* mRNA and IL-10 production by extra-intestinal DC (Manicassamy et al., 2009). Similarly, the TLR1/2-agonist Pam3CSK4 efficiently induced *Aldh1a2* in spleen-DC (Wang et al., 2011) (Figure 2).

In line with the previous data, MLN-DC from MyD88$^{-/-}$ mice exhibited decreased expression of *Aldh1a2* mRNA and RALDH activity (Guilliams et al., 2010; Wang et al., 2011) and an impaired capacity to induce gut-tropic T cells (Wang et al., 2011). However, a recent report did not find differences in RALDH activity between wild type and MyD88$^{-/-}$ MLN-DC (Molenaar et al., 2011). Although the reasons for these discrepant results remain to be determined, MLN-DC from mice lacking TLR2 (MyD88-dependent TLR) also showed decreased RALDH activity and impaired capacity for gut-homing induction (Wang et al., 2011).

Interestingly, RA did not efficiently induce *Aldh1a2* in MyD88$^{-/-}$ spleen-DC, which was correlated with a lack of RARβ in MyD88$^{-/-}$ DC, suggesting that MyD88 expression is also required for RA-mediated DC education (Villablanca et al., 2011b). Nonetheless, another study showed that daily RA treatment during *ex vivo* DC differentiation induced *Aldh1a2*

mRNA in MyD88$^{-/-}$ BM-DC (Feng et al., 2010). Since RA induces RARβ (Villablanca et al., 2011b), it is possible that prolonged RA treatment during BM-DC differentiation might induce RARβ in MyD88$^{-/-}$ DC, hence restoring their responsiveness to RA.

Although TLR signals are sufficient *in vitro*, and partially required *in vivo*, for gut-associated DC education, it is interesting to mention that MLN-DC from germ-free mice exhibited only a mild reduction in RALDH activity (Guilliams et al., 2010) and were able to induce α4β7 on T cells (Stagg et al., 2007). Although the latter study did not examine CCR9, it is possible that in the absence of gut microflora, food-derived and/or endogenous TLR agonists (Erridge, 2010) might still contribute to gut-associated DC conditioning.

5. Role of RA and gut-homing T cells in intestinal immune tolerance

5.1 Oral Immune Tolerance

Oral immune tolerance (OT) prevents pro-inflammatory responses to innocuous orally administered antigens, such as food and commensal microbiota, and it has been investigated as a potential treatment for diverse autoimmune conditions (Mayer and Shao, 2004; Weiner et al., 2011). Since patients with inflammatory bowel diseases (IBD) exhibit markedly decreased OT (Kraus et al., 2006; Kraus et al., 2004), impaired OT is believed to contribute importantly to the pathogenesis of IBD. However, despite being known for over 50 years (Chase, 1946), the mechanisms responsible for OT remain unclear (Weiner et al., 2011).

Administering high doses of oral antigen typically results in T cell clonal deletion and/or anergy (Chen et al., 1995; Van Houten and Blake, 1996), while low doses and repeating regimes of oral antigen administration lead to active immunosuppression by inducing Treg (Dubois et al., 2009; Hauet-Broere et al., 2003; Zhang et al., 2001). However, these two mechanisms are not mutually exclusive and some Treg also display characteristics of anergic T cells (von Boehmer, 2005).

Although orally administered antigens can be presented to T cells by DC in PP and MLN, it has been proposed that OT requires MLN (Spahn et al., 2002; Worbs et al., 2006) but not PP (Kraus et al., 2005; Spahn et al., 2001). In this setting, intestinal CD103^{+} DC take up antigens in the LP and, in a CCR7-dependent manner, migrate to MLN to activate naïve T cells (Worbs et al., 2006). Nevertheless, it is possible that the site for OT induction might also be determined by the nature of the antigen or its site of entry into the intestinal mucosa (Niedergang and Kweon, 2005). For instance, a prominent role for the liver has been proposed in OT models using haptens as a tolerizing agent (Dubois et al., 2009; Goubier et al., 2008; Yang et al., 1994), while tolerance to gliadin seems to rely on the spleen (Pre et al., 2011).

5.2 Immunological cellular network orchestrating OT

Antigen-specific T_{REG} can be readily observed in PP and MLN as early as 24–48h post oral feeding (Hauet-Broere et al., 2003; Zinselmeyer et al., 2005). Previous work indicated that nT_{REG} are not required for OT (Mucida et al., 2005), whereas iT_{REG} are critical for OT generation (Curotto de Lafaille et al., 2008; Hadis et al., 2011).

Other studies have highlighted a contribution of pDC in OT. PDCA-1^{+} pDC are recruited to the LP early after exposure to food proteins (Ohue et al., 2011). In fact, a subset of immature pDC expresses CCR9 (Hadeiba et al., 2008), which has been proposed to play a role in pDC homing to the gut (Wendland et al., 2007). Moreover, pDC carrying dietary antigens to the liver were required for OT by inducing anergy/deletion of effector T cells (Dubois et al., 2009; Goubier et al., 2008). In addition, although adoptive transfer of T_{REG} isolated from orally tolerized mice is sufficient to confer tolerance to non-tolerized mice (Broere et al.,

2008; Hauet-Broere et al., 2003), a recent study showed that pDC were also sufficient to transfer tolerance (Goubier et al., 2008). Thus, it will be important to determine whether the involvement of T_{REG} and/or pDC in OT might depend on the specific experimental system.

Intestinal macrophages also contribute to T_{REG} induction in an IL-10-dependent manner (Denning et al., 2007; Murai et al., 2009). Moreover, mice lacking F4/80 or CX3CR1 (the latter expressed in intestinal macrophages), exhibited impaired OT (Hadis et al., 2011; Lin et al., 2005). Given that $CX3CR1^+$ cells do not migrate to MLN (Schulz et al., 2009), these cells are likely to play a role in OT by contributing to IL-10 production and/or by promoting T_{REG} proliferation in the intestinal LP (Hadis et al., 2011). Interestingly, IL-10 is required for OT and for proper T_{REG} function in the gut mucosa (Cassani et al., 2011; Chaudhry et al., 2011; Murai et al., 2009).

5.3 Critical role of RA-dependent gut-homing T cells in OT

RA promotes T_{REG} differentiation and upregulates gut-homing receptors on T cells (Mora, 2008; Siewert et al., 2007), suggesting that RA and/or gut-homing receptors might be required for OT generation. In agreement with this possibility, we have recently found that OT is abrogated in VAD mice, in mice lacking CCR9, or upon MAdCAM-1 blockade (Cassani et al., 2011). Our data are in agreement with recently published work showing that OT is significantly impaired in mice lacking α4β7 or its endothelial ligand MAdCAM-1 (Hadis et al., 2011). It is possible that T_{REG} need to home to the small intestine LP to undergo further antigen-dependent expansion (Hadis et al., 2011). In addition, our data suggest that T_{REG} need to home to the small intestine LP to complete their functional differentiation and acquire IL-10-producing capacity, a process that requires IL-10 signaling as well as IL-10 production by gut-homing T_{REG} (Cassani et al., 2011).In fact, $IL\text{-}10^+$ T_{REG} are critical for immune tolerance at mucosal surfaces (Rubtsov et al., 2008) and IL-10 is needed for OT induction in different experimental models (Cong et al., 2004; Kraus et al., 2005; Navarro et al., 2011; Rizzo et al., 1999).

In line with our hypothesis that T_{REG} need gut-homing receptors to become IL-10-producing cells, OT induction in an asthma model was correlated with the presence of $CCR9^+$ $IL\text{-}10^+$ Treg in the lung (Navarro et al., 2011). In addition, $IL\text{-}10^+$ cells were found among circulating memory $CCR9^+$ T cells in healthy human volunteers, whereas memory $CCR9^{Neg}$ T cells did not produce IL-10 (Papadakis et al., 2003), suggesting that human T cells might also need to express gut-homing receptors to acquire tolerogenic IL-10-producing capacity.

Interestingly, a recent report showed that Th17 cells also need to home to the small bowel to become tolerogenic, a process that is mediated by the chemokine receptor CCR6 (Esplugues et al., 2011). Although this study did not assess the role of RA or α4β7/CCR9 in Th17 homing and/or tolerogenicity, both RA and α4β7/CCR9 also appear to be required for efficient Th17 location/differentiation in the gut (Cha et al., 2010; Wang et al., 2010). Nonetheless, it is also possible that different types of immune responses or inflammation might induce alternative homing mechanisms for T cell migration to the gut mucosa (Villablanca et al., 2011a).

Even though the data discussed above suggest that RA plays a critical role in mucosal immune tolerance, it was recently shown that RA and IL-15 cooperated to induce proinflammatory cytokines and pathogenic effector T cells in an experimental model of gluten-related enteropathy (DePaolo et al., 2011). Given that these effects were observed in mice overexpressing IL-15, the physiological implications of these observations remain to be determined. Nevertheless, since RA is also required for homing and/or differentiation of potentially proinflammatory Th17 cells in the gut mucosa (Cha et al., 2010; Wang et al.,

2010), it is possible that RA will have either a tolerogenic or pro-inflammatory role depending on the immunological context.

Moreover, some inflammatory conditions can affect gut-associated DC imprinting capacity. Induction of chronic experimental colitis decreased *Tgfb2* and *Aldh1a2* mRNA expression in $CD103^+$ MLN-DC, which correlated with impaired RALDH activity and decreased T_{REG} induction (Laffont et al., 2010). On the other hand, $CD103^+$ MLN-DC from patients with active Crohn's disease efficiently induced gut-tropic T cells (Jaensson et al., 2008), suggesting that intestinal inflammation does not necessarily lead to decreased RA production by DC. However, the latter study did not look at T_{REG} induction by MLN-DC from Crohn's patients and it could be that in a given inflammatory context induction of RA-dependent gut-tropic effector T cells is maintained, but T_{REG} generation is not.

6. RA as a "mucosal adjuvant"

Transcutaneous immunization (TCI) requires RA to generate efficient IgA immune responses in the gut, an effect that was ascribed to the induction of gut-tropic IgA-ASC (Chang et al., 2008). Moreover, gut-immune responses induced upon vaccination with adenovirus (Ad)-based vectors were impaired in $\beta7^{-/-}$ mice (lacking $\alpha4\beta7$) or in VAD mice (Kaufman et al., 2011). In fact, even mild to moderate VA deficiency significantly impaired vaccine-induced protection in the intestinal mucosa, a defect that was completely reversed by short-term VA supplementation (Kaufman et al., 2011). Thus, RA appears to be a critical factor for inducing efficient humoral and T cell immune responses in the intestinal mucosa.

It should be kept in mind that, besides inducing gut-tropic T cells, RA might have additional homing-independent effects on immune cells (e.g., on DC migration/maturation), which might contribute to boost immune responses in extra-intestinal compartments, such as lungs and vaginal mucosa (Martin Mdel et al., 2010; Tan et al., 2011).

Of interest, Ad-based vectors can also induce extra-intestinal expression of *Aldh1a1* and *Aldh1a2* enzymes, raising the possibility that ectopic RA production might contribute to induce gut-homing T cells at the site of immunization (Ganguly et al., 2011; Kaufman et al., 2011). However, analogous to subcutaneous immunization with vaccinia virus (Liu et al., 2006), it is likely that intramuscular immunization with Ad-based vectors also requires gut-associated lymphoid tissues (e.g., MLN) to imprint gut-tropic T cells.

Remarkably, oral or subcutaneous RA supplementation was sufficient to condition DC in skin-draining PLN to produce RA and to induce gut-tropic T and B cells in this extra-intestinal lymphoid compartment (Hammerschmidt et al., 2011; Villablanca et al., 2011b). Moreover, subcutaneous RA supplementation at the time of immunization elicited a potent IgA response in the small intestine, which protected mice from cholera toxin-induced diarrhea (Hammerschmidt et al., 2011). Therefore, given its capacity to efficiently induce gut-homing lymphocytes in extra-intestinal tissue, RA, or other pharmacological RAR-agonists, could potentially be used as "mucosal adjuvants" in the context of vaccination to enhance protective immune responses in the gut (Mora et al., 2008).

7. Concluding remarks

After the seminal observation identifying RA as the key factor to induce gut-homing T cells (Iwata et al., 2004), it was determined that RA modulates several other immunological properties in the intestinal mucosa, such as the induction of gut-tropic B cells, IgA-ASC, T_{REG}, and Th17 responses. It will be important to further define in which contexts RA promotes tolerogenic versus pro-inflammatory immune responses in the gut mucosa.

Regarding gut-associated DC education, several microenvironmental factors have been proposed to be important to confer DC with RA-producing capacity, and recent evidence suggests that GM-CSF, Wnt/β-catenin pathway, TLR signals, and RA itself might have a role in gut-associated DC education. Whether and how these conditioning factors interact with each other to educate intestinal DC remains to be determined.

In addition to enhance protective immune responses in the gut, recent evidence indicates that RA-dependent gut-homing T cells also play a key role in establishing, and probably maintaining, immune tolerance in the intestinal mucosa. Given that therapies blocking gut-homing receptors are currently in clinical use or in advanced clinical trial for IBD, it will be important to assess whether a chronic gut-homing blockade might paradoxically interfere with normal intestinal immune homeostasis.

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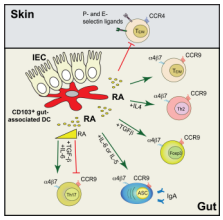

Figure 1. Retinoic acid orchestrates intestinal immunity

$CD103^+$ gut-associated-DC synthesize all-*trans* retinoic acid (RA), which is necessary and sufficient to induce gut homing receptors α4β7 and CCR9 on effector T and B cells, while reciprocally inhibiting the expression of skin homing receptors (P- and E-selectin ligands and CCR4). RA also potentiates the differentiation of $Foxp3^+$ regulatory T cells (requiring TGF-β) and Th2 cells (requiring Th2 polarizing cytokines). In addition, RA promotes the induction of IgA-ASC (also needing IL-5 and/or IL-6). Of note, RA seems to be required for the induction of intestinal Th17 cells, although medium to high RA concentrations have been shown to inhibit *ex vivo* Th17 differentiation. It is also unclear whether RA is needed for extra-intestinal Th17 responses. Green and red lines indicate induction or inhibition, respectively.

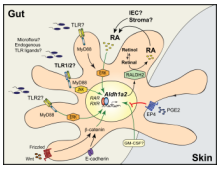

Figure 2. Mechanisms proposed to induce *Aldh1a2* in gut-associated DC

Aldh1a2 is the main RALDH isoform expressed in gut-associated DC, although *Aldh1a1* and *Aldh1a3* have also been described in other gut-associated cell types, such as IEC and stromal cells. Whereas the mechanisms controlling the expression of the latter RALDH isoforms remain to be determined, recent literature show that the expression of *Aldh1a2* in DC can be modulated by several microenvironmental stimuli. In particular, recent evidence indicates that RA itself is necessary and sufficient to induce its own synthesis by upregulating *Aldh1a2* in DC as a positive feedback loop. IEC are among the potential sources of RA to educate DC in the gut, although this remains to be demonstrated. In addition, several other factors have been proposed to induce *Aldh1a2* in DC, including MyD88-dependent TLR2 signals, Wnt/β-catenin pathway, GM-CSF, and PPARγ-agonists (the latter not shown in the figure). In contrast, PGE2 acting on EP4 receptor inhibits *Aldh1a2* induction in skin-associated DC. However, although the critical role of RA in *Aldh1a2* induction in gut-associated DC has been consistently shown by several groups, the contribution of other factors in physiological gut-associated DC education and the intracellular signaling pathways mediating these effects (including MyD88, ERK and JNK) are currently less clear. Green and red lines indicate induction or inhibition of *Aldh1a2* expression, respectively.